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Discovery of novel mammary developmental and cancer genes using ENU mutagenesis

The rate of death from breast cancer has only begun to fall in recent years. To dramatically reduce breast cancer death rates we need new therapeutic targets, currently a major challenge facing cancer researchers. This requires an understanding of the undiscovered pathways that operate to drive breast cancer cell proliferation, cell survival and cell differentiation, pathways which are also likely to operate during normal mammary development, and which go awry in cancer. The discovery of signalling pathways operative in breast cancer has utilised examination of mammary gland development following systemic endocrine ablation or viral insertion, positional cloning in affected families and the investigation of genes identified in other tissues for activity in the mammary gland. . A systematic search for genes controlling mammary development and cancer will make these new targets available for new drug development. To date there has been no systematic search for genes controlling mammary development.

This project seeks to discover new genes involved in mammary gland carcinogenesis, by screening for mammary gland defects and tumors in the pedigrees produced by the large scale recessive ENU mutagenesis project at the John Curtin School Australian National University

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Introduction

To date there has been no systematic search for genes controlling mammary development and carcinogenesis, and this project seeks to undertake the first such search by participation in the large scale recessive ENU mutagenesis project currently being conducted by Prof. Chris Goodnow, at the John Curtin School, Australian National University (ANU).

Functional characterisation of genetic loci is urgently required if the imminent completion of the mouse genome is to be of immediate benefit. This requirement is being met in a number of ways, including seven large scale ENU mutagenesis projects under way worldwide. Of these, just three are undertaking genome wide recessive screens. Recessive mutations, requiring the loss of function of two alleles, provide far greater insight into the genetic control of normal development and carcinogenesis than dominant, gain-of-function mutations, but require more complex breeding of pedigrees. This has been achieved at the ANU, where the established program of ENU mutagenesis has recently received significant financial support from the Australian Federal Government ($A11.5 million Major National Research Facilities Grant) and The Australian National University ($A8 million) for the construction of expanded animal handling facilities. Many granting bodies are supporting the use of this facility in addition to the CDMRP. For example the National Institutes of Health USA recently awarded Prof Goodnow a $US 5 million RO1 entitled "Genes for Tolerance and Immunity. Using an inbreeding scheme, ENU mutagenised mouse pedigrees are produced so that on average each pedigree carries 100 defective genes, every animal carries 12 homozygous functional mutations, and 3 animals per pedigree will carry null mutations for the same gene. This is being undertaken using both wild type backgrounds and sensitising mono- and bi-transgeneic backgrounds. To date 20 phenotypes have had the causative mutations successfully mapped.

We adopted this technology early in its evolution at the ANU and screened the pedigrees that were produced by the first round of mutagenesis. Screening was undertaken using whole mount histology of the 4th inguinal mammary gland. Approximately 1100 mice were analysed, and a number of abnormalities and tumors were identified. From these 6 pedigrees were selected for propagation. Male siblings of affected females from these 6 selected pedigrees were used to recreate the pedigrees using the same 3 generation inbreeding scheme. Members of these pedigrees were tested by mammary biopsy to identify affected individuals that could then be used to establish stable breeding lines and begin the mapping crosses.

Body

Work during the first 12 months of this award covered Task 1 in the Statement of Work.

Task1a. We chose 6 pedigrees to pursue, based on the nature of the defect and available resources. The details of each are shown in Figure 1. These pedigrees all show increased ductal side branching in addition to a second lesion, mostly multiple invasive lesions or ductal carcinoma in situ. When we examined the remaining glands from an affected animal we generally observed the same phenotype, arguing against a chance occurrence.

Task 1b-e Regeneration and aging of selected pedigrees with mammary tumors from genetic back-ups to confirm the genetic basis of the observed defects. This was successfully undertaken for the 6 chosen pedigrees. The brothers of the females used in the first screen were used to recreate each pedigree. Multiple males were used given the 1 in 2 chance that they carried the mutation. Each pedigree was recreated using a 3 genereation inbreeding scheme. Multiple brothers were mated to wild type females and the female progeny were mated with their fathers. The female progeny of father daughter matings were then aged for screening. Approximately 30 females per pedigree were tested by mammary biopsy.

Biopsy testing.

The essential difficulty here was that tumors may not develop until after the animal has passed its useful breeding age. To address this problem we relied on the association between tumors and increased ductal side branching (Figure 2), reasoning that if this is a precursor lesion then it will be apparent at an earlier age. We have also examined the glands very carefully as tumors may be very small. We chose to examine the 180 animals from the six pedigrees at either 5 months of age and at 8 months of age using mammary biopsy. Examples of the results are shown in Figure 3 and the results are summarized in Figure 4. In three of the six pedigrees we identified single small tumors where large multiple tumors were seen in the original pedigree. These animals were selected for further breeding, but their progeny failed to reproduce the large tumors seen in the original screen. It is apparent from these results that we failed to regenerate the original tumor phenotypes in any of the 6 pedigrees chosen for propagation.

Task 3 and Task 4. These tasks were not undertaken because of our failure to reproduce the original tumor phenotypes in the 6 pedigrees that we regenerated.

We believe that our failure to reproduce the tumor phenotypes detected in the original screen was due to the multiple mutations required for tumor formation. In the original screen the ENU provided a sensitizing mutation, and the environment the subsequent mutations required for tumorigenesis. By definition these

subsequent hits occurred within a short time frame to allow the detection of tumors at 8 months. When we attempted to recapitulate these pedigrees we believe that although the ENU mutation was present, the other hits did not occur within the time frame allowed by the necessity to breed from affected animals.
We had expected that a single mutation provided by ENU would provide sufficient sensitization to tumor formation for success, but the experiment has shown us otherwise. This failure has led us to redesign our methodology to include a sensitizing event in the background, allowing ENU to provide a second hit. Due to our failure to recapitulate the original phenotypes we cannot proceed with the research contract as initially approved. We therefore request a modification of the research project and statement of work as detailed below.

Request for a modification to the Research Plan and Statement of Work

Our failure to regenerate the six selected pedigrees requires us to revise our research plan and its statement of work. We believe that introduction of a transgenic oncogene into the background will enable ENU to provide a second hit in the carcinogenic process. We believe, based on the proven enhancement of carcinogenesis by combined oncogenes (such as myc and ras), that this approach will allow us to regenerate pedigrees with enhanced tumorigenesis due to the ENU-induced loss of tumor suppressor gene function.

We investigated the constitutively active C3-SV40T transgenic model as a prototype transgenic model. The issue with such models was whether the pregnancy-induced decrease in tumor latency, which is seen in most mammary specific transgenic models due to the sensitivity of the MMTV promoter to pregnancy hormones, would hamper successful breeding of our pedigrees. We examined 58 breeding pairs held at the Garvan Institute. Twelve (21%) females were unable to wean pups due to tumor burden following the first pregnancy. Of the 36 animals examined following a second pregnancy, 27 (75%) were unable to wean pups. Ethical considerations, of <10% tumor burden, generally precluded a third mating. This investigation demonstratred that constitutive transgene expression would greatly hamper the breeding of pedigrees and the mapping crosses, especially as we would expect tumor latency to decrease in the context of an ENU-induced loss of a tumor suppressor gene.

An inducible transgene system is required. Such a system has recently been constructed in the laboratory of Dr Lewis Chodosh. It utilizes the MMTV promoter to drive the expression of the reverse tetracycline-dependent transactivator, resulting in mammary specific expression of the tet-operator controlled transgene in response to treatment with tetracyclin. This model is insensitive to the hormonal state of the animal and shows no tumorigenic activity in the absence of doxycycline (see appendix paper 1). We know from our experience to date that a single ENU-induced mutation does not hamper the breeding of pedigrees, and so use of this system, where transgene activity is induced for screening, but remains off for animal propagation, offers us the best method of achieving our aims.

The transgene of choice would induce tumors with a latency of six months or more, allowing us to find animals harboring homozygous ENU induced mutations due to significantly decreased latency. The transgene

should also have direct relevance to human breast cancer. Ideally, many of the tumors would regress once transgene expression was turned off. All these criteria are met by the tet-operator-myc transgene model developed by the laboratory of Dr Lewis Chodosh (see appendix paper 2).

Thus we propose a modified Statement of Work that includes the use of this bi-transgeneic animal. We have established a collaboration with Dr Lewis Chodosh to make these animals available to us. The importation and establishment of the line will reduce the amount we can achieve in the remaining 2 year time frame. We therefore request a 1 year no cost extension to allow us to complete much of what we have originally proposed with the new mouse model in the next 3 years.

Proposed Revision of the Statement of Work

2003-2005

Task 1.
Months 1 to 9.
1. Establish Tet-myc bitransgenic mice at ANU by rederivation.
2. Establish genotyping PCRs and test.

Task 2. Establish and screen 200 inbred pedigrees
Months 9 to18.
a. Mate homozygous FVB Tet-myc bitransgenic females with 200 C57Bl6 ENU mutagenised males to establish 200 pedigrees. Keep G1 males. Simultaneously mate 20 unmutagenised males to establish 20 control pedigrees.
b. Mate G1 males to homozygous Tet-myc bitransgenic females to produce G2 offspring.
c. Mate G2 females with their fathers to produce 25 G3 females per ENU or control pedigree.
d. Induce transgene expression at 9 weeks of age with doxycycline. Monitor G3 females for mammary tumors by palpitation every 10 days. Select females with tumors from the ENU group which become palpable before tumors are seen in the control group.

Task 3. Establish pedigrees from selected females.
Months 12-24
a. Stop doxycycline treatment and mate tumor bearing female with father, or brother if father fails to breed. Test progeny for accelerated tumorigenesis and breed pedigree to homozygosity. Examine tumor phenotype to prioritize pedigrees for mapping.

Task 4. Map mutations
*Months 24-*36.
a. Mate homozygous females to male FVB to produce F2
b. Phenotype F2 and collect DNA into affected (20 animals) and unaffected (20+ animals) pools.
c. Genotype using 100 micro satellite markers polymorphic between FVB and C57Bl6 chosen throughout the genome.
d. Identify locus and regenotype using 100 locus specific polymorphic markers.
e. Continue intercross/backcross if no informative recombinations are found.
f. Identify candidate genes at a sub 1 cM locus from the mouse genome map. Exclude those not expressed in the mammary gland. Begin sequencing of remaining candidates to identify ENU-induced mutation.

We are confident that this approach will overcome the unforeseen problems encountered during our first attempt to adapt this new and powerful technology to cancer research, and will identify new tumor suppressor genes active in the mammary gland.

Key research accomplishments

• Recreation of six pedigrees from male siblings of females screened in the first round using an inbreeding scheme.

• Biopsy screening of these animals at 5 and 8 months of age, with the identification of females showing increased ductal side branching and in one case a tumor. Breeding of these animals in an attempt to establish stable breeding lines.

• Investigation of alternative approaches to overcome the problems encountered during the first experiment, and the identification of a suitable transgenic system to pursue our objectives.

• Modification of our research strategy to overcome the problems encountered.

Reportable outcomes

No reportable outcomes

Conclusions

So What?

If we are successful we will discover new tumor suppressor genes that are active in the mammary gland. This will have direct relevance to breast cancer, providing potential markers of prognosis and new targets for therapy.

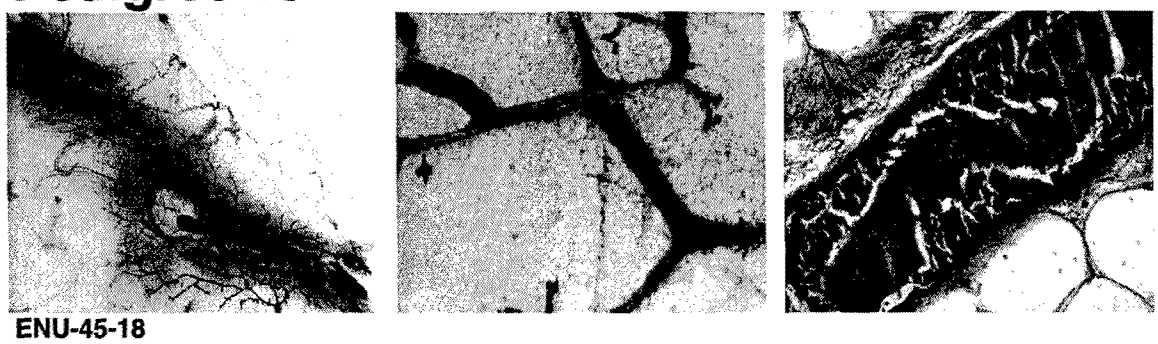
Appendices

Figures 1-3

Paper 1. Gunther et al. FASEB J 16:283-292 2002
Paper 2. D'Cruz et al. Nature Medicine 7:235-239 2001

Figure 1 (next 3 pages). Pedigrees chosen for mapping.

Pedigree 45



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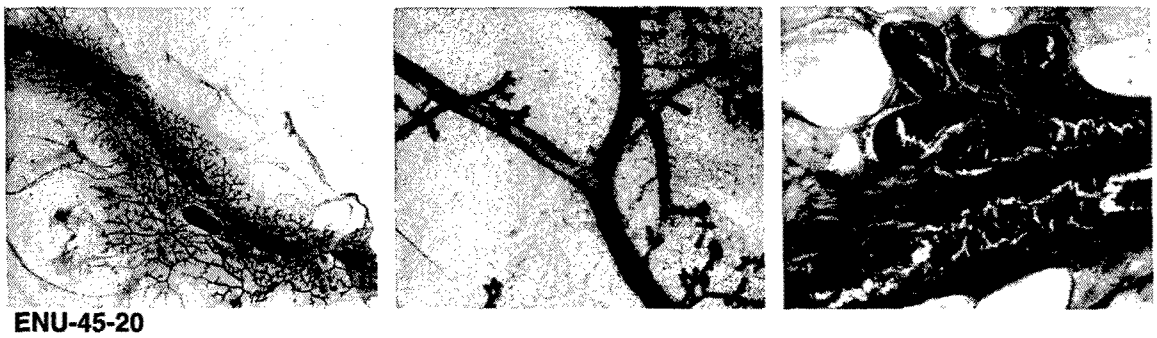

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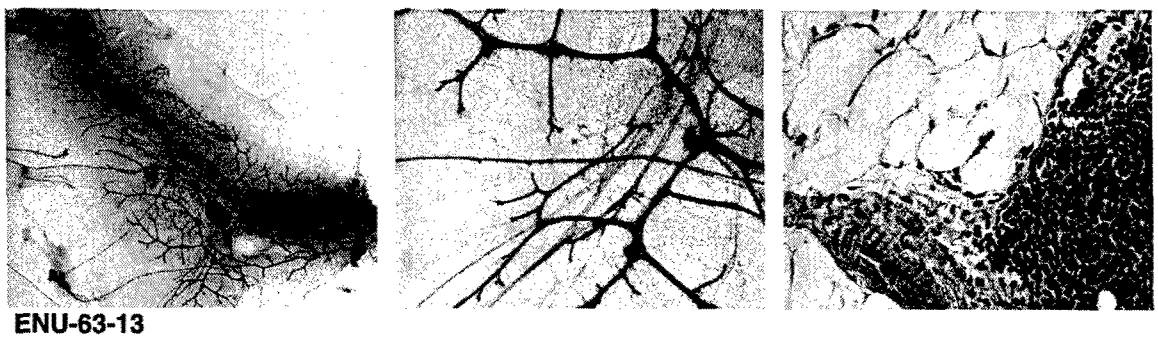
Pedigree 63

ENU-63-11



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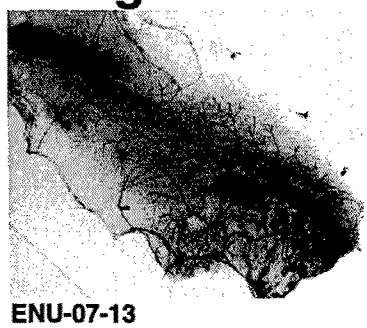
Pedigree 07

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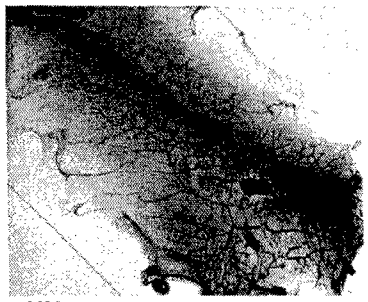
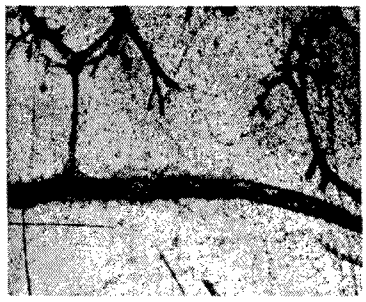
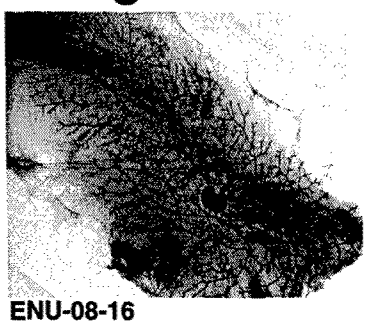
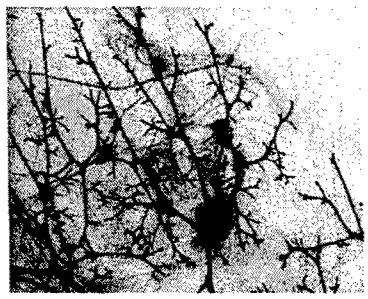
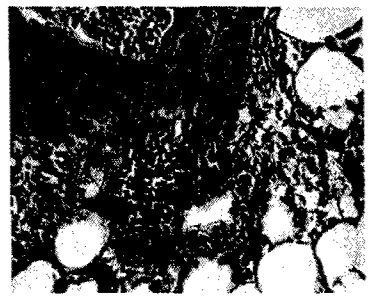



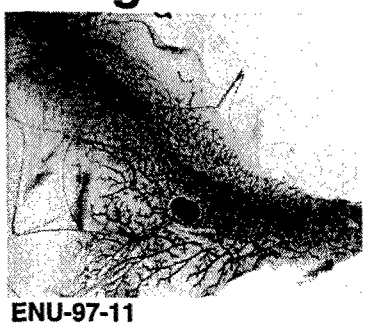
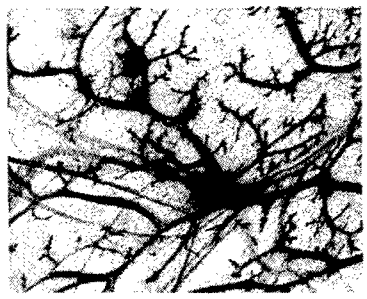
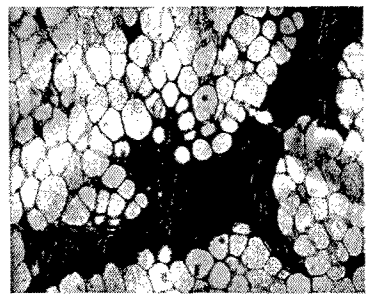

ENU-07-35

Pedigree 08

ENU-08-16

Pedigree 97

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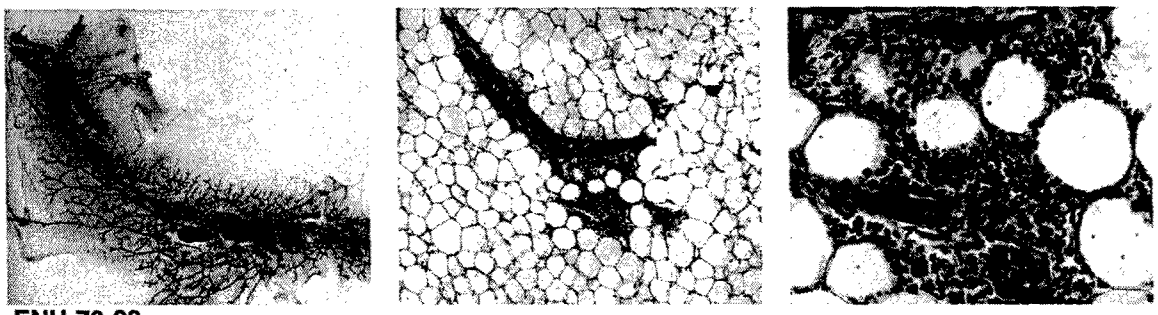
Pedigree 70

ENU-70-22



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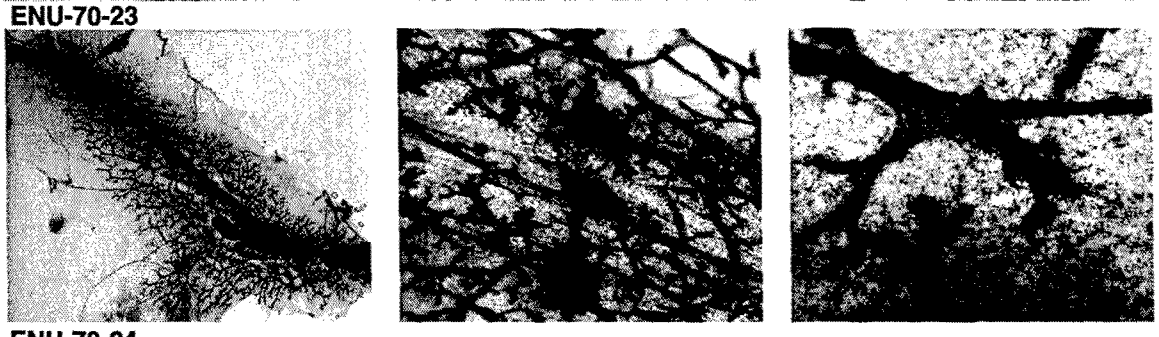

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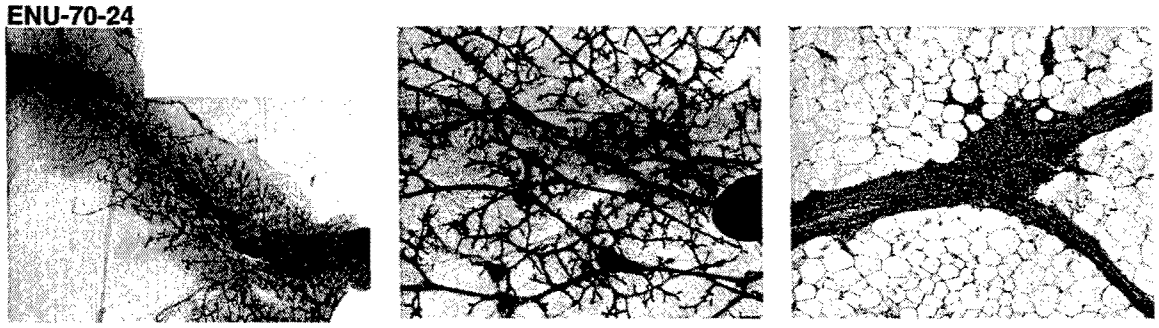

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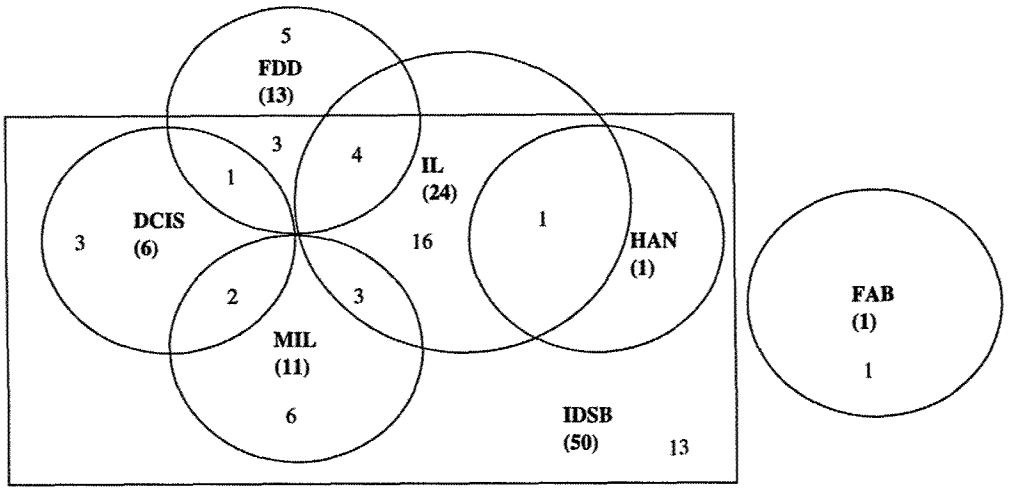
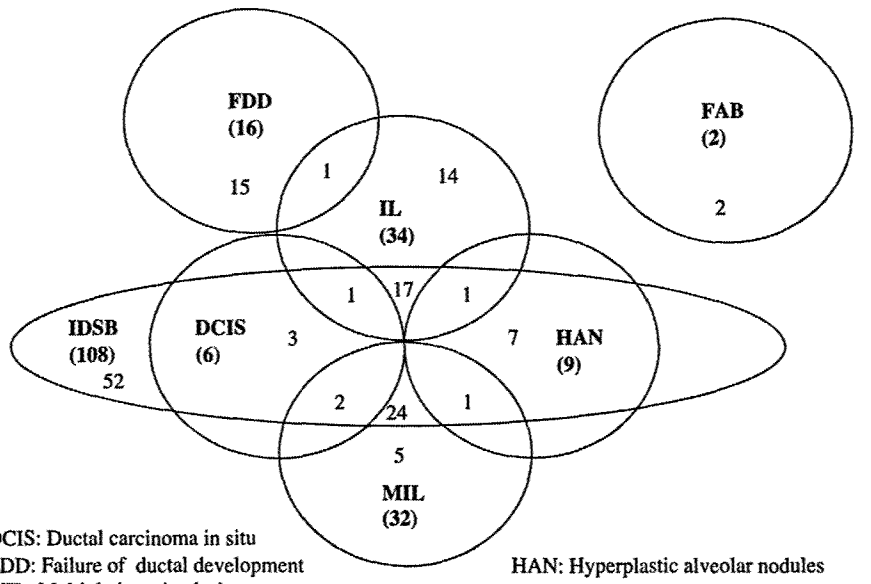
Figure 2. Association of phenotypes found in the first screen



DCIS: Ductal carcinoma in situ
FDD: Failure of ductal development
MIL: Multiple invasive lesions
IL: Invasive lesion
HAN: Hyperplastic alveolar nodules
IDSB: Increased ductal side branching
FAB: Failure of alveolar bud development

Phenotype frequency

Number of pedigrees with individual(s) displaying defects.



DCIS: Ductal carcinoma in situ
FDD: Failure of ductal development
MIL: Multiple invasive lesions
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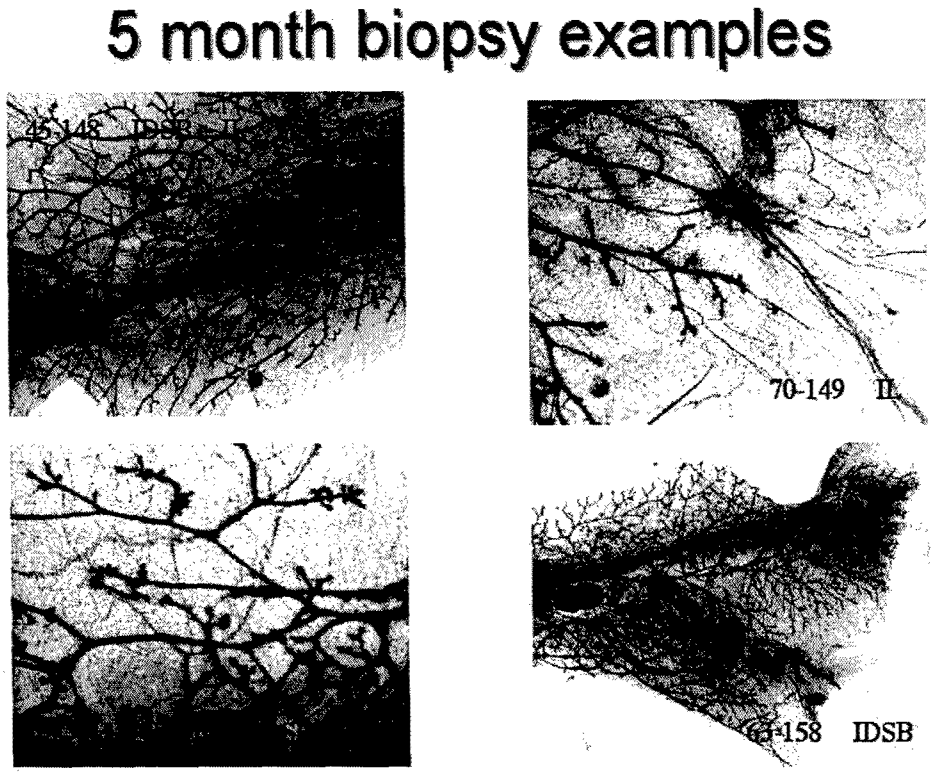
HAN: Hyperplastic alveolar nodules
IDSB: Increased ductal side branching
FAB: Failure of alveolar bud development

Associated phenotypes

Number of glands with multiple phenotypes.

Figure 3. Examples of defects found in the regenerated pedigrees.



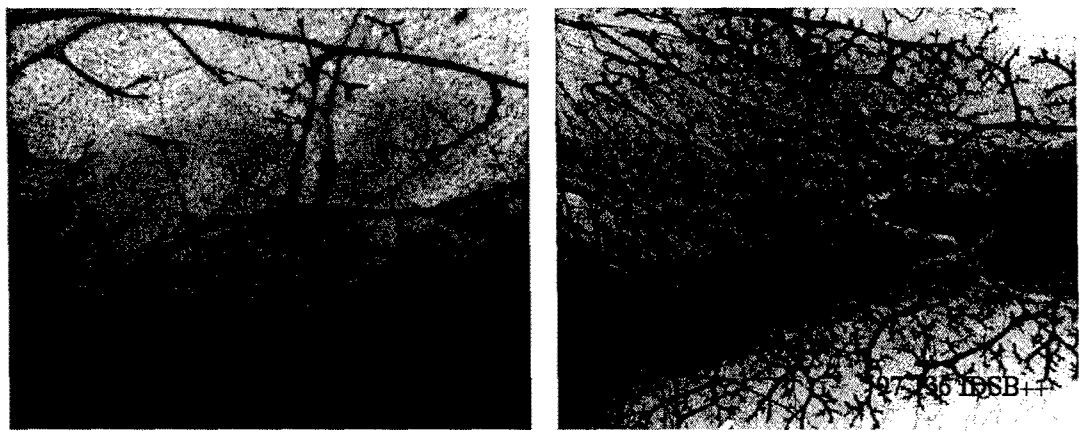


Figure 4. Summary of results from regeneration of pedigrees.

	SCREENING 8 Months		BIOPSY 5 or 8 Months	
45	45-20	DCIS+IDSB+TEBS+MIL	45-148	IDSB+ IL
	45-18	DCIS	45-	IDSB multiple
63	63-11	HAN+IDSB	63-	IDSB multiple
	63-13	MIL	63-110	IL
7	7-35	IDSB	7-	IDSB multiple
	7-13	DCIS		
70	70-22	IDSB+MIL		
	70-23	IDSB+MIL		
	70-24	IDSB+MIL	70-149	IL
	70-26	IDSB+MIL (bp)	70-	IDSB multiple
8	8-16	IDSB+MIL	8-	IDSB multiple
			8-176	IDSB+TEBS
97	97-11	IDSB+MIL	97-139	IDSB++
			97-	IDSB multiple

Abbreviations and terms used in figures.

DCIS	Ductal Carcinoma In Situ
IDSB	Increased Ductal Side Branching
IDSB Multiple	Many animals in pedigree exhibiting IDSB
TEBS	Aberrant Terminal End Buds
MIL	Multiple Invasive Lesions
MIL (bp)	Multiple Invasive Lesions at branch points
IL	Single Invasive Lesion

A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology

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ABSTRACT **Normal developmental events such as puberty, pregnancy, and parity influence the susceptibility of the mammary gland to tumorigenesis in both humans and rodent model systems. Unfortunately, constitutive transgenic mouse models that rely on mammary-specific promoters to control transgene expression have limited utility for studying the effect of developmental events on breast cancer risk since the hormonal signals governing these events also markedly influence transgene expression levels. A novel transgenic mouse system is described that uses the MMTV-LTR to drive expression of the reverse tetracycline-dependent transactivator *rtTA*. Transgenic mice expressing rtTA in the mammary epithelium were crossed with reporter lines bearing tet operator-controlled transgenes. We tested the ability to spatially, temporally, and quantitatively control reporter gene expression after administration of doxycycline to bitransgenic mice. Transgene expression using this system can be rapidly induced and deinduced, is highly mammary specific, can be reproducibly titrated over a wide range of expression levels, and is essentially undetectable in the uninduced state. Homogeneous transgene expression throughout the mammary epithelium can be achieved. This system permits transgene expression to be restricted to any desired stage of postnatal mammary gland development. We have developed a mammary-specific, doxycycline-inducible transgenic mouse model for studying the effect of mammary gland development on transgene-mediated phenotypes. Unlike other mammary-specific, transgenic systems that have been described, this system combines spatially homogeneous transgene expression in the mammary epithelium during puberty, pregnancy, lactation, and involution with the use of an orally administered, inexpensive, and widely available inducing agent. This system offers new opportunities for the transgenic analysis of mammary gland biology in vivo.—Gunther, E. J., Belka, G. K., Wertheim, G. B. W., Wang, J., Hartman, J. L., Boxer, R. B., Chodosh, L. A. A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. *FASEB J.* 16, 283–292 (2002)**

Key Words: transgenic mice · MMTV · transgene induction

EPIDEMIOLOGIC AND ANIMAL studies strongly suggest that the susceptibility of the mammary gland to carcinogenesis is a function of the gland's developmental state at the time of exposure to oncogenic stimuli (1–6). Reproductive endocrine events such as puberty, pregnancy, and parity each influence the susceptibility of the mammary gland to the subsequent development of cancer. The molecular and cellular mechanisms by which normal developmental events modulate breast cancer risk are unknown. Understanding these mechanisms will require increasing our understanding of the interaction between mammary development, reproductive history, and oncogenic pathways.

Postnatal development of the mouse mammary gland closely resembles that of the human and provides a unique and powerful model for studying the relationship between developmental biology and cancer (7, 8). For example, the mammary-specific expression of several oncogenes implicated in human breast cancer in transgenic mice has provided confirmation of both their tumorigenic potential and their ability to disrupt normal programs of epithelial differentiation (9–12). Nevertheless, although constitutive mammary-specific transgenic models have proved valuable, the utility of these models for probing the effect of reproductive events on breast cancer risk has been limited by the characteristics of available mammary-specific promoters (13). For instance, the mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters are hormonally regulated and are therefore markedly up-regulated during pregnancy and lactation (14, 15). As a result, reproductive events in these models alter transgene expression and breast cancer risk, thereby precluding any meaningful analysis of the effect of reproductive history on cancer susceptibility.

Recently, inducible transgenic mouse models have been described for mammary-specific transgene expression. In the first reported system of this type, the MMTV-LTR was used to drive expression of the tet-responsive transactivator tTA. In the absence of tetra-

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cycline, tTA is able to activate expression of a second transgene controlled by tet operator sequences (16). This system has demonstrated the time-dependent reversibility of SV40 T antigen-induced salivary gland hyperplasias and BCR-ABL-induced leukemias in mice (17, 18).

However, the utility of this system for studying mammary gland biology may be limited by mosaic transgene expression since a relatively small fraction of mammary epithelial cells demonstrate detectable reporter gene expression (16). In addition, many tissues other than the mammary gland exhibit moderate levels of transgene induction in this model. More recently, an MMTV-driven, inducible transgenic model that uses a transactivator comprised of a modified ecdysone receptor has been described (19). This model has been shown to yield homogeneous transgene expression in the mammary epithelium during lactation in the presence of the plant ecdysteroid inducer ponasterone A. The level and spatial distribution of transgene expression during other stages of mammary gland development have not been reported. Unlike tetracycline-based systems, however, this system uses an inducer that is not yet commercially available and requires parenteral administration.

We reasoned that transgenic mouse models suitable for analyzing the effect of development on breast cancer susceptibility would require the ability to deliver tightly regulated, mammary-specific transgene expression during any stage of postnatal mammary gland development. Ideally, such a system would permit homogeneous transgene expression in the mammary epithelium, titratable transgene expression levels, and rapid kinetics of induction and deinduction. As described below, we believe we have created a doxycycline-inducible mouse model system that fulfills these criteria. By permitting both the timing and level of transgene expression to be varied experimentally in a variety of developmental contexts, this model offers new opportunities for studying mammary gland biology in vivo.

MATERIALS AND METHODS

Transgenic mice

To create the plasmid pMMTV-*rtTA*-pA, the full-length *rtTA* minigene (1.0 kb fragment from plasmid pUHD172-neo, a gift from Dr. Henry Bujard) was subcloned into pBluescript II KS (Stratagene, San Diego, CA) downstream of a 2.9 kb fragment containing the promoter elements derived from plasmid, pMMTV-polyoma MT (a gift from Dr. Philip Leder) (20). This promoter includes 1.2 kb of sequence upstream of the transcriptional start site of the MMTV-LTR and 0.6 kb of leader sequence from v-H-ras; 1.8 kb of SV40 sequence carrying splicing and polyadenylation signal sequences was subcloned downstream of the *rtTA* gene. The reporter construct pTetO-LacZ was created by subcloning the *LacZ* gene from plasmid pUHG16–3 (a gift from Dr. Bujard) downstream of a CMV minimal promoter and seven adjacent tet operator sites derived from pTet-Splice (Gibco-BRL, Rockville, MD) and upstream of the SV40 splicing and polyadenylation signal sequences above (20). The construct pTetO-Luc is identical to pTetO-LacZ except that the *LacZ* gene has been replaced by the firefly luciferase coding region excised from pGL3 (Promega, Madison, WI).

Restriction fragments containing each transgene were isolated from vector sequences and prepared for microinjection into fertilized oocytes. All transgenic lines were created on an inbred FVB/N background. Potential founders were identified by screening genomic DNA from tail biopsies for the presence of the transgene using the polymerase chain reaction. Amplification reactions for genotyping animals used the following oligonucleotide pairs: MMTV-*rtTA*-pA: 5′-ATCCG-CACCCTTGATGACTCCG-3′ and 5′-GGCTATCAACCAACA-CACTGCCAC-3′ to amplify a 349 bp segment spanning the junction of MMTV-LTR and v-H-ras leader sequences; Tetop-LacZ: 5′-GGTCTGGAC ACCAGCAAGGAGCTGC-3′ and 5′-GCGCATCGTAACCGTGCATCTGCC-3′ to amplify a 307 bp sequence in the LacZ gene; Tetop-Luc, 5′-CACGAAATT-GCTT CTGGTGGC-3′ and 5′-TCGAAGATGTTGGGGTGT-TGG-3′ to amplify a 469 bp sequence in the luciferase gene. Reaction conditions were 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s.

A founder line carrying construct MMTV-*rtTA*-pA was designated MTB and characterized by crosses with reporter strains. Bitransgenic mice were derived from crosses between mice hemizygous for each transgene except for the TetO-LacZ founder line TZA, which was bred to homozygosity and used in the generation of some MTB/TZA and TZA mice. As observed for many transgenic mouse lines, male and female mice bred to homozygosity at the *MTB* locus were noted to be poor breeders. In addition, homozygous MTB dams often fail to raise their litters to weaning age. MTB hemizygous dams show normal fertility and support litter sizes typical of wild-type FVB/N mice. A small fraction of MTB females became ill (hunched appearance, weight loss) after prolonged administration of doxycycline (>10 months). These mice were killed when moribund and were noted at dissection to have a thickened intestinal wall with evidence of a lymphoid infiltrate consistent with lymphoma. This illness has not been seen in a comparable cohort of wild-type FVB mice on chronic doxycycline or in MTB mice in the absence of doxycycline.

Transgene expression was induced in mice by replacing normal drinking water with 5% sucrose containing doxycycline. For prolonged inductions, doxycycline-containing water was changed every 3 days. Mice were mated between 4 and 8 wk of age. The day a vaginal plug was detected was defined as the first day of pregnancy. Pregnancy time points were confirmed by examination of embryos at the time of death. Regression time points were harvested after the forced weaning of pups on day 12 of lactation.

β-galactosidase solution assay

Harvested mouse mammary glands were snap frozen on dry ice and stored at −80°C. Protein extracts were prepared essentially as described (21). Approximately 500 mg of frozen tissue was homogenized in 1.0 ml of buffer (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 500 mM sucrose, 150 mM NaCl, 10 mM dithiothreitol) using a polytron homogenizer. Homogenates were cleared by two centrifugation steps performed at 12,000 *g* for 20 min at 4°C. The soluble fraction was transferred to a fresh tube and protein concentration was quantitated by the method of Bradford. ONPG (*o*-nitrophenyl-B-D-galactopyranoside) was used as a colorimetric substrate in a standard β-galactosidase assay (21); 10–30 μg of protein was assayed in replicate reactions that were terminated at increasing time points. The optical density of each reaction was measured at

420 nm and values were plotted against time to determine the reaction rate.

Histochemical staining of tissue sections

Mammary glands harvested for in situ determination of β-galactosidase activity were frozen in OCT (Miles Laboratories, Tarrytown, NY). Tissue blocks were stored at −80°C. Freshly cut tissue sections were applied to glass slides, prefixed in 0.5% glutaraldehyde, rinsed twice in PBS at room temperature for 20 min, and stained for β-galactosidase activity at 37°C in staining solution containing 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal) at 1 mg/ml, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, and 1 mM $MgCl_2$. After staining, slides were rinsed twice for 20 min in PBS at RT, postfixed in 0.5% glutaraldehyde, rinsed in PBS, and coverslipped.

Luciferase assay

Snap-frozen tissues were homogenized in Passive Lysis Buffer (Promega) using a dounce homogenizer. Homogenates were cleared by centrifugation at 12,000 *g* and the supernatant was assayed for protein concentration by the method of Lowry. Luciferase activity was measured using the Dual Luciferase Assay Kit (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) according to manufacturer's instructions.

Mammary gland morphology

Mammary glands were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Whole mounts of mammary glands were prepared and stained with carmine alum as described (22). Sections were applied to glass slides and stained with hematoxylin and eosin (H&E).

RESULTS

Expression of rtTA in mammary glands of transgenic mice

Transgenic founder lines were generated harboring the construct MMTV-*rtTA*-pA in which expression of the reverse tetracycline transactivator rtTA ('Tet-On') is driven by the MMTV-LTR. This construct contains a portion of the v-H-ras leader sequence, which has been associated with enhanced mammary expression of transgenes downstream of the MMTV-LTR (11). Although MMTV-promoter-based transgenes typically are expressed maximally during late pregnancy and lactation, the MMTV-*rtTA* founder line MTB displayed easily detectable *rtTA* expression levels in the mammary glands of 5-wk-old virgin female mice (**Fig. 1*A***). Expression of *rtTA* was relatively uniform among age-matched

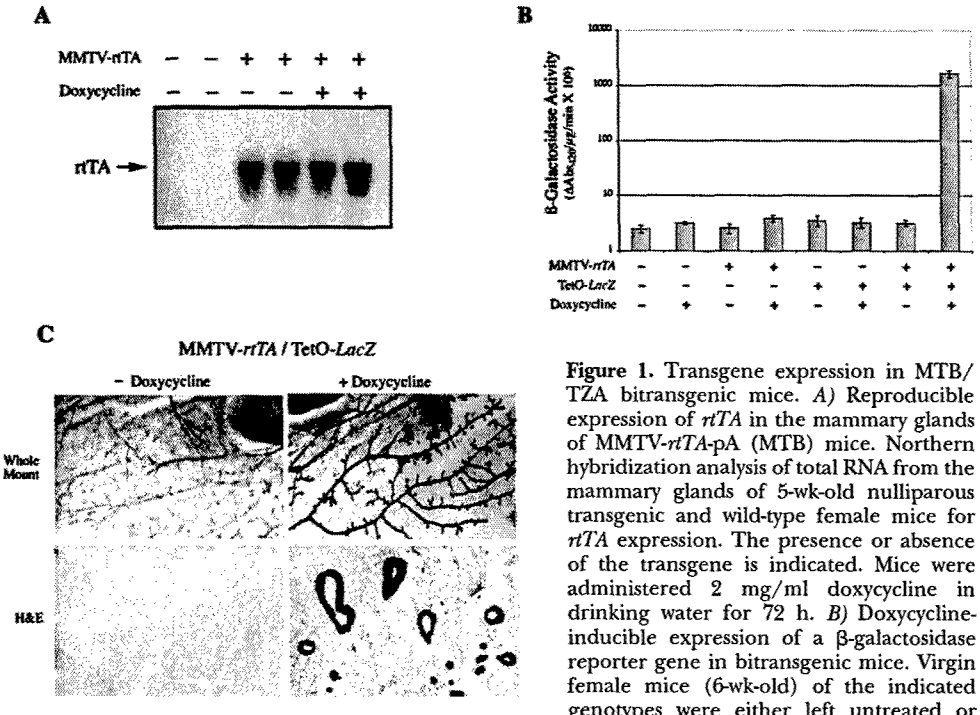

Figure 1. Transgene expression in MTB/TZA bitransgenic mice. *A)* Reproducible expression of *rtTA* in the mammary glands of MMTV-*rtTA*-pA (MTB) mice. Northern hybridization analysis of total RNA from the mammary glands of 5-wk-old nulliparous transgenic and wild-type female mice for *rtTA* expression. The presence or absence of the transgene is indicated. Mice were administered 2 mg/ml doxycycline in drinking water for 72 h. *B)* Doxycycline-inducible expression of a β-galactosidase reporter gene in bitransgenic mice. Virgin female mice (6-wk-old) of the indicated genotypes were either left untreated or administered 2 mg/ml doxycycline in drinking water for 72 h. Mammary gland extracts were prepared and assayed for β-galactosidase activity. *C)* Homogeneous, mammary epithelial-specific β-galactosidase activity in doxycycline-treated MTB/TZA mice: 6-wk-old virgin female bitransgenic mice were either left untreated (left panels) or induced with doxycycline (right panels) as above. Note homogeneous staining of mammary epithelium, including a terminal end bud present at the 12 o'clock position in the bottom right panel. Mammary glands were whole mounted (upper panels) or embedded in OCT and sectioned (lower panels) before histochemical staining for β-galactosidase activity.

virgin transgenic mice and, as expected, was unperturbed by the administration of doxycycline (Fig. 1*A*).

Inducible transgene expression is tightly regulated and spatially homogeneous

To determine the ability of the MTB line to permit inducible transgene expression, reporter mice were generated harboring a TetO-LacZ transgene comprised of the bacterial *LacZ* gene downstream of a minimal promoter containing a heptamer of tet operator sequences. MTB mice were crossed with mice of the TetO-LacZ-bearing transgenic line TZA. Wild-type, MTB, TZA, and MTB/TZA bitransgenic nulliparous female mice were either induced by administration of 2 mg/ml doxycycline in their drinking water for 72 h or were left untreated. Mammary gland-derived protein extracts were then assayed for β-galactosidase activity. Neither uninduced MTB/TZA glands nor induced MTB or TZA glands yielded β-galactosidase activity above the background levels observed in wild-type FVB glands (Fig. 1*B*). In contrast, mammary extracts prepared from doxycycline-treated MTB/TZA mice exhibited β-galactosidase activity levels ~500-fold above background. These results indicate that transgene expression in this system is highly inducible and tightly regulated.

Heterogeneous transgene expression in the mammary epithelium has often been observed in MMTV-based transgenic mouse models (11, 16). As a result, transgene-mediated phenotypes may have low penetrance and may reflect effects of transgene expression on selected subsets of cells rather than on the epithelium as a whole. To determine the spatial localization of transgene expression in our system, in situ histochemical staining for β-galactosidase activity was performed on whole-mounted mammary glands harvested from doxycycline-induced MTB/TZA bitransgenic mice (Fig. 1*C*). This analysis confirmed induction of β-galactosidase activity in doxycycline-treated bitransgenic animals as well as the lack of detectable β-galactosidase activity in uninduced bitransgenic animals. Analysis of both whole mounts and sections demonstrated that induction of β-galactosidase activity in 6-wk-old MTB/TZA female mice is confined to the mammary epithelial tree and is remarkably homogeneous within the mammary epithelium (Fig. 1*C*). Homogeneous β-galactosidase expression was also demonstrated in terminal end buds (Fig. 1*C*), which are highly proliferative structures that drive ductal elongation during puberty and may be particularly sensitive to oncogenic stimuli. Histochemical staining of sections failed to detect β-galactosidase activity in uninduced MTB/TZA glands or monotransgenic genetic controls (Fig. 1*C* and data not shown).

Transgene induction is mammary specific

Northern hybridization analysis of tissues derived from 6-wk-old virgin MTB mice demonstrated high levels of *rtTA* expression in the mammary gland (**Fig. 2*A***). Consistent with other MMTV transgenic models, lower levels of expression were observed in the salivary gland and the male seminal vesical (Fig. 2*A* and data not shown). Expression of *rtTA* mRNA was not detected in any other tissue examined. These findings suggest that *rtTA* transgene expression in MTB mice may exhibit a degree of mammary specificity greater than that typically seen in MMTV transgenic models (23).

To further investigate tissue specificity in this model system, transgenic mice carrying the luciferase gene under the control of tet-operator sequences (TetO-Luc) were generated and crossed to MTB mice. Assays to detect luciferase gene expression are more sensitive and have a greater dynamic range than assays for β-galactosidase activity. Bitransgenic MTB/TetO-Luc mice were induced for 72 h with 2 mg/ml doxycycline.

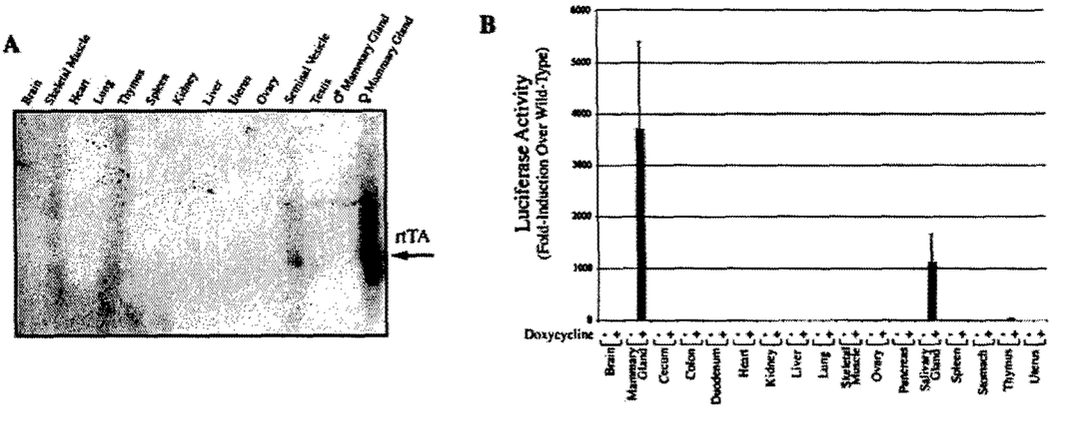

Figure 2. Mammary-specific transgene induction in bitransgenic mice. *A)* Mammary-specific expression of *rtTA* in MTB mice. Northern hybridization analysis of total RNA from tissues harvested from 6-wk-old virgin MTB mice for *rtTA* expression. *B)* Doxycycline-inducible expression of a luciferase reporter in tissues from bitransgenic MTB/TetO-Luc mice. A panel of 17 tissues was harvested from uninduced and induced 6-wk-old nulliparous female MTB/TetO-Luc bitransgenic mice. Equal amounts of protein from the tissues indicated were analyzed for luciferase activity and compared with values obtained from wild-type littermates. Mice were induced with 2 mg/ml doxycycline administered in drinking water for 72 h before tissue harvest.

Genetic and uninduced controls were analyzed in parallel. Luciferase assays were performed on protein samples from a panel of 17 tissues and normalized to protein concentration. These studies demonstrated the doxycycline-dependent 3000-fold induction of luciferase activity in the mammary glands of MTB/TetO-Luc bitransgenic mice as well as somewhat lower levels of induction in the salivary glands (Fig. 2*B*). Low but detectable levels of induced expression were observed in the thymus, a tissue shown to express MMTV-driven transgenes (23). Remarkably, neither mammary gland, salivary gland, nor thymus demonstrated detectable luciferase activity in the absence of doxycycline induction (Fig. 2*B*). These findings confirm that this bitransgenic system is both mammary specific and tightly regulated by doxycycline.

Titratable levels of transgene expression

A principal advantage of an inducible expression system is that it permits the titration of transgene expression to a desired level (20, 24). The ability to regulate transgene expression levels in the mammary epithelium is required for studying how the level of transgene expression affects mammary gland phenotype and for achieving comparable levels of transgene expression during different developmental stages. Accordingly, the ability to reproducibly titrate transgene expression levels in the mammary gland was tested by generating a dose-response curve for reporter gene induction in MTB/TZA mice. Nulliparous female MTB/TZA bitransgenic mice were administered increasing doxycycline doses via drinking water for 72 h before being killed. Protein extracts prepared from harvested mammary glands were analyzed for β-galactosidase activity (**Fig. 3*A***). Induction of β-galactosidase activity in MTB/TZA mice was first detectable at a doxycycline concentration of 0.03 mg/ml and was near maximal at 0.5 mg/ml. Intermediate doses of doxycycline reproducibly induced intermediate levels of β-galactosidase activity. These data demonstrate that this doxycycline-dependent transgenic system permits transgene expression to be titrated to a desired level.

Rapid induction of transgene expression

The ability to analyze the short-term effects of transgene induction on normal tissues requires the ability to rapidly induce transgenes. Moreover, precise timing of transgene induction is critical for studying the effect of transgene expression on developmental processes. The kinetics of *LacZ* transgene induction in MTB/TZA bitransgenic mice was determined by measuring β-galactosidase activity in the mammary glands of nulliparous females after exposure to doxycycline (Fig. 3*B*). As observed earlier, no β-galactosidase activity was detected in uninduced MTB/TZA glands above the background activity levels measured in wild-type glands. In contrast, β-galactosidase activity was first detected in MTB/TZA animals 6 h after doxycycline exposure.

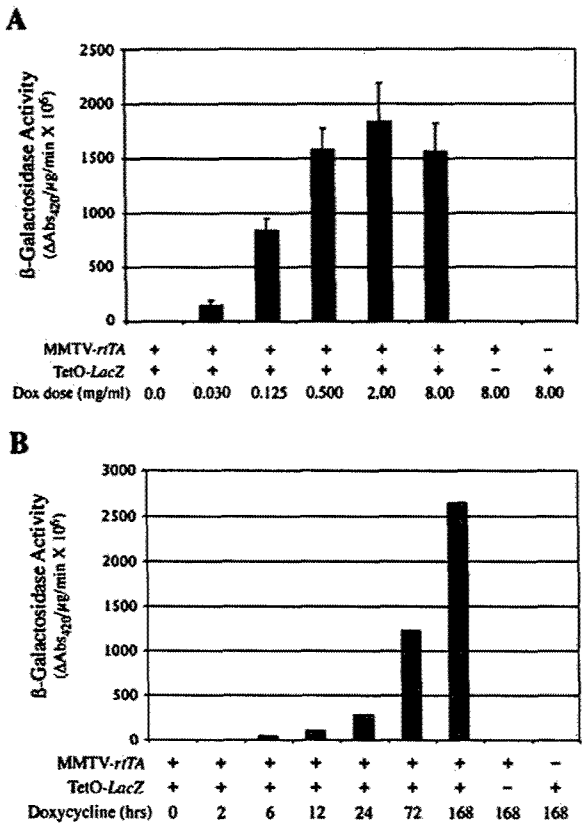

Figure 3. Dose-response and kinetics of transgene induction. *A)* Dose-response curve for doxycycline-inducible reporter gene expression: 6-wk-old nulliparous female MTB/TZA bitransgenic mice and monotransgenic controls were administered doxycycline in drinking water supplemented with 5% sucrose for 72 h before tissue harvest. Mammary gland protein extracts were assayed for β-galactosidase activity. *B)* Kinetics of doxycycline-inducible reporter gene expression. 6-wk-old nulliparous female MTB/TZA bitransgenic mice and monotransgenic controls were administered 2 mg/ml doxycycline in drinking water before tissue harvest. Mammary gland protein extracts were assayed for β-galactosidase activity. Doxycycline administration was performed by orogastric gavage of bitransgenic animals in the case of the 2 h point.

β-Galactosidase activity continued to increase with increasing times of doxycycline exposure up to 1 wk, most likely as a consequence of the long half-life of the *LacZ* mRNA transcript and encoded β-galactosidase protein. In contrast, MTB mice bitransgenic for an inducible c-MYC transgene attain steady-state levels of c-MYC expression within 48 h of induction, presumably reflecting the short half-life of the c-MYC mRNA and protein (ref 25 and data not shown).

The ability to turn off transgene expression is also a desirable property of inducible systems. The kinetics of transgene deinduction would be expected to depend on the half-life of the mRNA and protein encoded by the transgene, and the rate at which doxycycline levels decline in vivo after its discontinuation. Analysis of MTB/TetO-MYC mice has demonstrated that c-MYC transgene expression levels decline to baseline levels

within 24 h after doxycycline withdrawal (ref 26 and data not shown).

Homogeneous transgene expression during multiple stages of postnatal mammary development

To characterize the magnitude and spatial pattern of transgene induction during other stages of postnatal mammary development, β-galactosidase activity was analyzed in the mammary glands of MTB/TZA bitransgenic female mice at developmental stages representing puberty, pregnancy, lactation, and postlactational involution. MTB/TZA females and genetic controls were induced with 2 mg/ml doxycycline for 72 h before mammary gland harvest. As before, no β-galactosidase activity was detected in uninduced mammary glands from MTB/TZA mice above that measured in wild-type and monotransgenic MTB and TZA glands. β-Galactosidase activity was highly induced in mammary glands from doxycycline-treated bitransgenic mice at each developmental stage analyzed (**Fig. 4*A***). The magnitude of induction of β-galactosidase activity in bitransgenic mice ranged from several 100-fold in nulliparous mice to several 1000-fold in pregnant and lactating mice. However, given that uninduced β-galactosidase levels are indistinguishable from background levels found in wild-type mammary glands, these estimates represent lower limits. Our finding that the levels of β-galactosidase activity present in pregnant and lactating glands of MTB/TZA mice exceeded the levels detected in virgin glands is consistent with the finding that *rtTA* expression is higher during pregnancy and lactation (Fig. 4*A* and data not shown).

A major limitation of mammary-specific transgenic models, particularly those using the MMTV-LTR, has been marked spatial heterogeneity of transgene expression within the mammary epithelium. Even though our data indicate that transgene expression is relatively homogeneous in the mammary glands of 6-wk-old mice, we wanted to characterize the spatial distribution of transgene expression at other stages of postnatal development. This was achieved by histochemical staining for β-galactosidase activity in frozen sections from induced MTB/TZA mice. Homogeneous, epithelial-specific staining was observed in glands harvested from induced MTB/TZA bitransgenic females during pu-

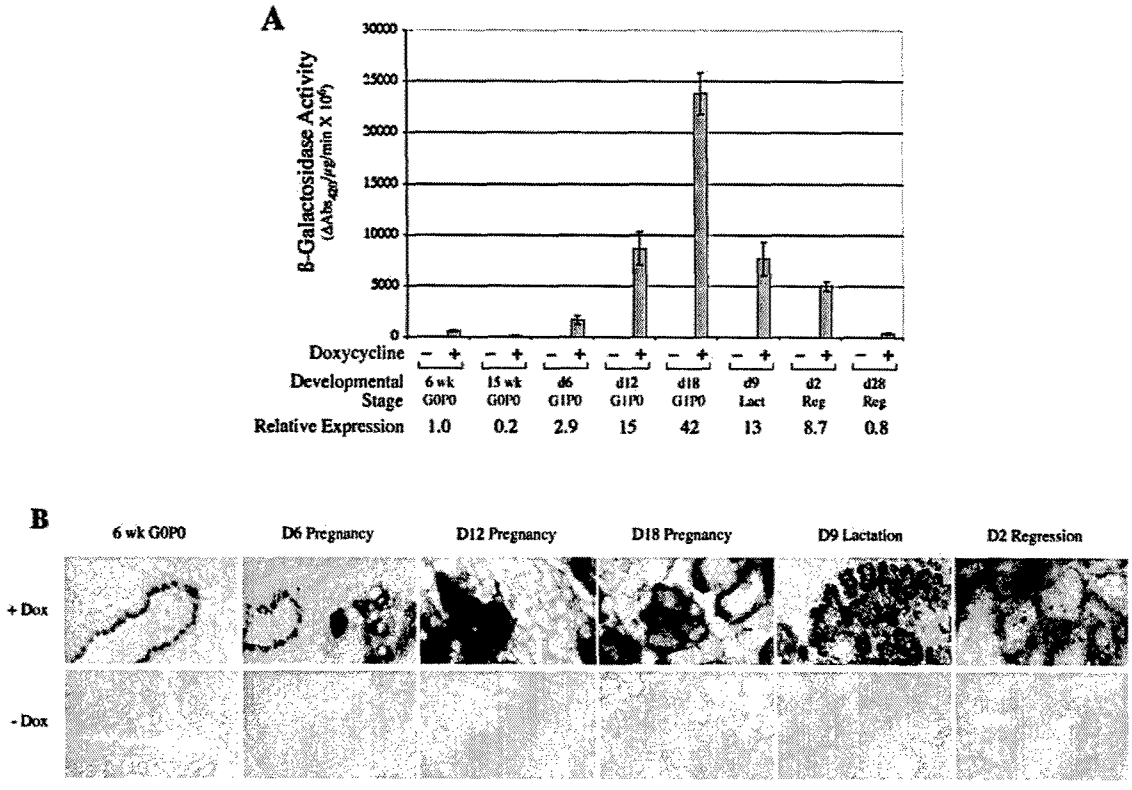

Figure 4. Developmental dependence of doxycycline-inducible reporter gene expression. Bitransgenic MTB/TZA mice or monotransgenic TZA mice were either left untreated or induced with 2 mg/ml doxycycline in drinking water for 72 h before tissue harvest. *A)* β-Galactosidase activity levels assayed in mammary gland protein extracts prepared from mice of the indicated genotypes and doxycycline exposure at eight stages of postnatal development. Values are shown for 6-wk-old and 15-wk-old nulliparous (G0P0) mice, as well as for mice on days 6, 12, and 18 of pregnancy (G1P0), day 9 of lactation (LACT), and days 2 and 28 of postlactational regression (REG). *B)* Frozen sections of OCT-embedded mammary glands of the induced and uninduced bitransgenic mice above were histochemically stained for β-galactosidase activity using X-gal.

berty, pregnancy, lactation, and postlactational involution (Fig. 4*B*).

Because the mammary epithelial compartment increases in size during pregnancy, direct comparison of β-galactosidase activity in mammary gland homogenates from MTB/TZA mice at different developmental stages can be problematic even after normalizing activity to protein levels. It is worth noting that histological sections from induced bitransgenic glands from pregnant and lactating mice exhibited more intense staining for β-galactosidase activity than comparable sections from virgin mice analyzed in parallel (Fig. 4*B* and data not shown). This suggests that induced β-galactosidase activity is greater on a per cell basis in the mammary epithelium of pregnant and lactating MTB/TZA mice vs. virgin animals.

Heterogeneous transgene expression in aging mice

Absolute levels of LacZ transgene expression were observed to decrease in MTB/TZA bitransgenic mammary glands harvested from 15-wk-old vs. 6-wk-old mice (**Fig. 5**). Consistent with this, expression of *rtTA* in the mammary glands of MTB mice decreases with age (data not shown). To determine the cellular basis for this change, 15-wk-old nulliparous MTB/TZA mice were analyzed for transgene expression by histochemical staining of mammary gland sections. Unlike other stages of mammary gland development analyzed, staining for β-galactosidase activity in this group of bitransgenic mice was heterogeneous, with ~10–20% of mammary epithelial cells exhibiting detectable activity (Fig. 5). This suggests that the decrease in reporter transgene expression in aging mice may in part be a consequence of expressing the transgene in only a subset of mammary epithelial cells. A similar heterogeneous pattern of β-galactosidase staining was also noted in mammary glands from15-wk-old bitransgenic mice that had undergone pregnancy, lactation, and 4 wk of postlactational involution. It is unclear whether unstained cells fail to express the transgene or express the transgene at a level below the limits of histochemical detection.

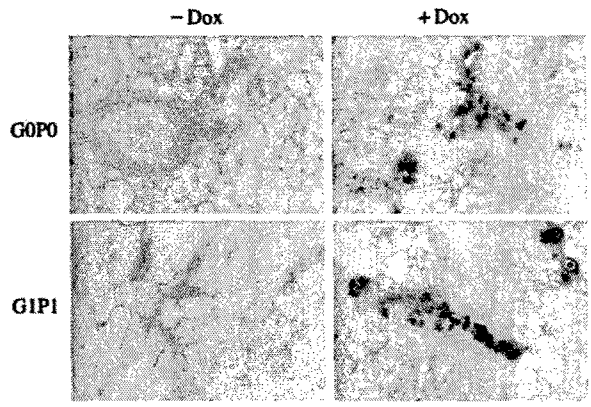

Figure 5. Heterogeneous reporter gene expression in mammary glands of 15-wk-old mice. Frozen sections of OCT-embedded mammary glands from uninduced and doxycycline-induced 15-wk-old nulliparous (G0P0, top panels) and parous (G1P1, bottom panels) bitransgenic MTB/TZA females were histochemically stained for β-galactosidase activity. Doxycycline-induced mice were administered 2 mg/ml doxycycline in 5% sucrose as drinking water for 72 h before tissue harvest.

Heterogeneous expression of the *LacZ* reporter transgene could be a direct consequence of gene silencing events at the *TZA* locus or an indirect consequence of gene silencing events at the *MTB* locus. We favor the former possibility, since *rtTA* transcript levels in the mammary gland are only slightly decreased in 15-wk-old induced MTB/TZA bitransgenic mice compared with 5-wk-old animals. In contrast, *LacZ* transcript levels induced in MTB/TZA are markedly decreased in 15-wk-old vs. 5-wk-old mice (data not shown).

Postnatal mammary development appears normal in MTB transgenic mice

For the MTB bitransgenic system we created to be of maximum utility, mammary development in these mice must be demonstrably normal. This criterion is important given concerns that overexpression of a strong transcriptional transactivator such as rtTA may be toxic to mammary epithelial cells (27). To address this issue, mammary glands from MTB and wild-type mice were analyzed morphologically for evidence of developmental abnormalities by examination of carmine-stained whole mounts and H&E-stained sections (**Fig. 6**). The highly ordered nature of the mammary epithelial tree permits this type of analysis to detect relatively subtle changes in gland development. These studies demonstrate that at both morphological (Fig. 6*A*) and histological (Fig. 6*B*) levels, mammary development in MTB animals is indistinguishable from that observed in wild-type mice at each stage of postnatal development, including puberty, pregnancy, lactation, and postlactational involution.

To determine whether high levels of rtTA expression alter mammary epithelial proliferation, BrdU incorporation in the mammary epithelium was measured during puberty in MTB mice. Levels of BrdU incorporation were indistinguishable between cohorts of MTB and wild-type age-matched virgin female mice (data not shown). MTB hemizygous and FVB/N wild-type dams were also indistinguishable with regard to the number of pups per litter and to the growth rate of pups (data not shown). These findings suggest that in addition to being morphologically normal, mammary development is functionally normal in MTB hemizygous mice.

DISCUSSION

This report describes a novel inducible mouse model system that permits spatially homogeneous transgene expression in the mammary epithelium of bitransgenic

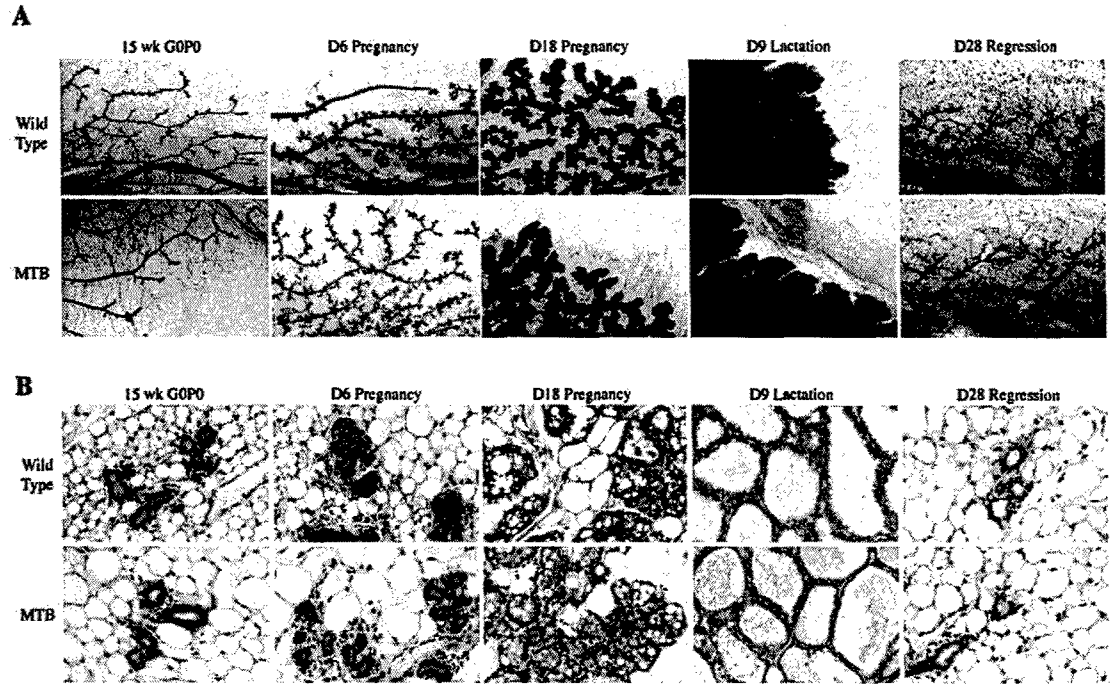

Figure 6. Mammary gland morphology in MTB mice during development. Mammary glands were harvested from MTB transgenic and wild-type female mice at various times of development. For analysis of whole-mount morphology (*A*), inguinal mammary glands were spread on slides, fixed, and subjected to carmine staining (7). For analysis of tissue histology, H&E-stained sections (*B*) from paraffin-embedded mammary glands were analyzed by light microscopy.

mice treated with doxycycline. This system allows regulatory molecules to be inducibly expressed in the mammary epithelium for a defined period of time, at a desired level, and during any stage of postnatal mammary development after treatment with a widely available, inexpensive, and easily administered inducing agent. Transgene expression is mammary specific, can be rapidly induced and deinduced, can be reproducibly titrated over a wide range of expression levels, and is essentially undetectable in the uninduced state. These features make this system ideally suited for expressing regulatory molecules in a spatially and temporally restricted manner during defined stages of mammary development or carcinogenesis.

A significant advantage of the tetracycline-inducible model described here is its spatially homogeneous pattern of transgene expression during multiple stages of postnatal mammary development. Nevertheless, our observation that older bitransgenic animals exhibit heterogeneous transgene expression highlights the importance of investigating the properties of transgene expression during each of the developmental stages at which transgene effects will be analyzed and of documenting that each of the stages of mammary development occurs normally in induced and uninduced mice expressing only the transcriptional transactivator.

Novel experimental approaches to the transgenic analysis of mammary epithelial biology are facilitated by the ability to inducibly control transgene expression. For example, the MTB transactivator-bearing transgenic line described in this report has been crossed to a second transgenic mouse line carrying a c-MYC transgene under the control of tet operator sequences. Inducible expression of c-MYC using this model system results in the formation of invasive mammary adenocarcinomas in a manner that is rapid, highly penetrant, mammary specific, and absolutely dependent on transgene induction by doxycycline (26). In the absence of doxycycline induction, c-MYC transgene expression is undetectable, and uninduced bitransgenic animals display a normal mammary phenotype. Removal of the oncogenic stimulus by transgene deinduction revealed that approximately half of adenocarcinomas arising as a result of dysregulated c-MYC expression remain dependent on transgene expression for maintenance; the other half acquire the ability to grow in the absence of c-MYC overexpression. Nearly 50% of tumors induced by c-MYC were found to carry spontaneous activating point mutations in *Kras2* or *Nras*, and the presence of such mutations was highly correlated with the ability of MYC-induced tumors to grow in a MYC-independent manner. These studies highlight the experimental opportunities that arise from the ability to abrogate transgene expression.

Amplification and overexpression of oncogenes, such as *c-MYC* and *ERBB2*, is found in a subset of human breast cancers (28, 29). However, little is known about the dose-response relationship between oncogene expression levels and mammary epithelial cell phenotype. The ability to titrate transgene expression

levels as described here should permit graded levels of oncogene expression to be achieved in the mammary epithelium of genetically identical mice. Moreover, tightly regulated temporal control over transgene expression will permit the results of oncogene activation to be analyzed in the normal epithelium of adult mice. This in turn will permit the restriction of oncogene activation to any stage of postnatal mammary development, thereby facilitating analysis of the effect of reproductive events on oncogene-mediated phenotypes.

Finally, additional experimental strategies to which this system can be applied include those aimed at using inducible expression of the cre recombinase for tissue-specific, conditional gene targeting. Doxycycline-inducible cre expression has been used to conditionally delete loxP-flanked gene segments in the mouse mammary epithelium (30). However, use of the WAP promoter to drive *rtTA* expression in this model required that gene deletion take place during lactation. Moreover, even under optimal conditions, cre-mediated recombination occurred in a small fraction of epithelial cells. Though mosaic gene deletion may be desirable in some experimental contexts, it is disadvantageous in others, particularly those designed to study the effect of gene deletion on development (31). Experiments are under way to examine whether the MTB transgenic line will permit more homogeneous cre-mediated deletion in the mammary epithelium in a manner that is less dependent on hormones of pregnancy.

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c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous *Kras2* mutations

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Although the process of mammary tumorigenesis requires multiple genetic events, it is unclear to what extent carcinogenesis proceeds through preferred secondary pathways following a specific initiating oncogenic event. Similarly, the extent to which established mammary tumors remain dependent on individual mutations for maintenance of the transformed state is unknown. Here we use the tetracycline regulatory system to conditionally express the human *c-MYC* oncogene in the mammary epithelium of transgenic mice. *MYC* encodes a transcription factor implicated in multiple human cancers. In particular, amplification and overexpression of c-MYC in human breast cancers is associated with poor prognosis, although the genetic mechanisms by which c-MYC promotes tumor progression are poorly understood[1,2]. We show that deregulated c-MYC expression in this inducible system results in the formation of invasive mammary adenocarcinomas, many of which fully regress following c-MYC deinduction. Approximately half of these tumors harbor spontaneous activating point mutations in the *ras* family of proto-oncogenes with a strong preference for *Kras2* compared with *Hras1*. Nearly all tumors lacking activating *ras* mutations fully regressed following c-MYC deinduction, whereas tumors bearing *ras* mutations did not, suggesting that secondary mutations in *ras* contribute to tumor progression. These findings demonstrate that c-MYC-induced mammary tumorigenesis proceeds through a preferred secondary oncogenic pathway involving *Kras2*.

We mated transgenic mouse lines expressing the reverse tetracycline-dependent transcriptional activator (rtTA) under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR; mouse line named *Tg(rtTA)1lach* called here MTB), to a second transgenic line containing exons 2 and 3 of human *MYC* under the control of the tetracycline-dependent minimal promoter (tetO; mouse line designated *Tg(MYC)1lach* called here TOM)[3,4]. Recent analysis of female MTB mice demonstrated high levels of rtTA mRNA expression in the mammary gland and salivary gland, and little or no detectable expression in other tissues (E. Gunther, submitted manuscript). Administration of doxycycline to MTB/TOM, MTB, TOM and non-transgenic littermates resulted in high levels of *MYC* transgene expression only in MTB/TOM bitransgenic female mice (Fig. 1*a*). Mammary tissue from uninduced bitransgenic littermates showed no transgene expression indicating that this system is tightly regulated. Finally, ornithine decarboxylase (*Odc*) mRNA levels increased 2.2-fold (Fig. 1*a*), and growth arrest and DNA-damage-inducible (*Gadd45a*) mRNA levels decreased 50% (data not shown), in induced bitransgenic females compared with uninduced controls demonstrating that the c-MYC transgene encodes a functional protein[5–8].

Morphological analysis showed that the mammary glands of MTB/TOM mice induced with doxycycline for 30 days were hyperplastic compared with either uninduced MTB/TOM or induced MTB control mice (Fig. 1*b*). Consistent with this, immunohistochemistry using antibodies specific for BrdU revealed a greater than 10-fold increase in BrdU incorporation in the mammary epithelium of induced bitransgenic females compared with control mice ($P < 0.001$; Fig 1*b*). Similarly, TUNEL analysis of mammary tissue demonstrated a greater than 10-fold increase in TUNEL-positive epithelial cells in induced bitransgenic mice compared with controls ($P < 0.001$; Fig. 1*b*). Together, these data indicate that c-MYC overexpression in the mammary epithelium increases both proliferation and apoptosis.

Compared with MTB/TOM mice induced with doxycycline for 30 days, the mammary glands of bitransgenic mice induced for 4 months displayed more severe morphological abnormalities, including focal hyperplastic, atypical lobuloalveolar growths referred to here as dysplasia (Fig. 1*c*). In contrast, uninduced bitransgenic mice maintained normal mammary gland morphology (Fig. 1*c*) indistinguishable from that found in wild-type mice (data not shown). We next determined whether *MYC* transgene overexpression is required for the maintenance of hyperplastic lesions in MTB/TOM mice. We treated bitransgenic mice with doxycycline for 30 weeks (chronically induced) and examined non-tumor-bearing glands from these mice 12 weeks after doxycycline withdrawal. Mammary glands from chronically induced bitransgenic mice had numerous epithelial hyperplasias and dysplasias, but most epithelial ducts from de-induced bitransgenic mice were histologically normal, indicating that hyperplastic and dysplastic mammary lesions that develop as a consequence of transgene overexpression remain dependent on c-MYC for their maintenance (Fig. 1*d*).

Consistent with the histological changes observed in chronically induced MTB/TOM mice, bitransgenic females developed mammary tumors with high penetrance (86%, $n = 57$) following an average of 22 weeks of induction (Fig. 2*a*). MTB mice chronically treated with doxycyline ($n = 15$), as well as untreated MTB ($n = 20$), TOM ($n = 18$) and MTB/TOM ($n = 10$) mice, exhibited normal histology of mammary tissues and did not develop tu-

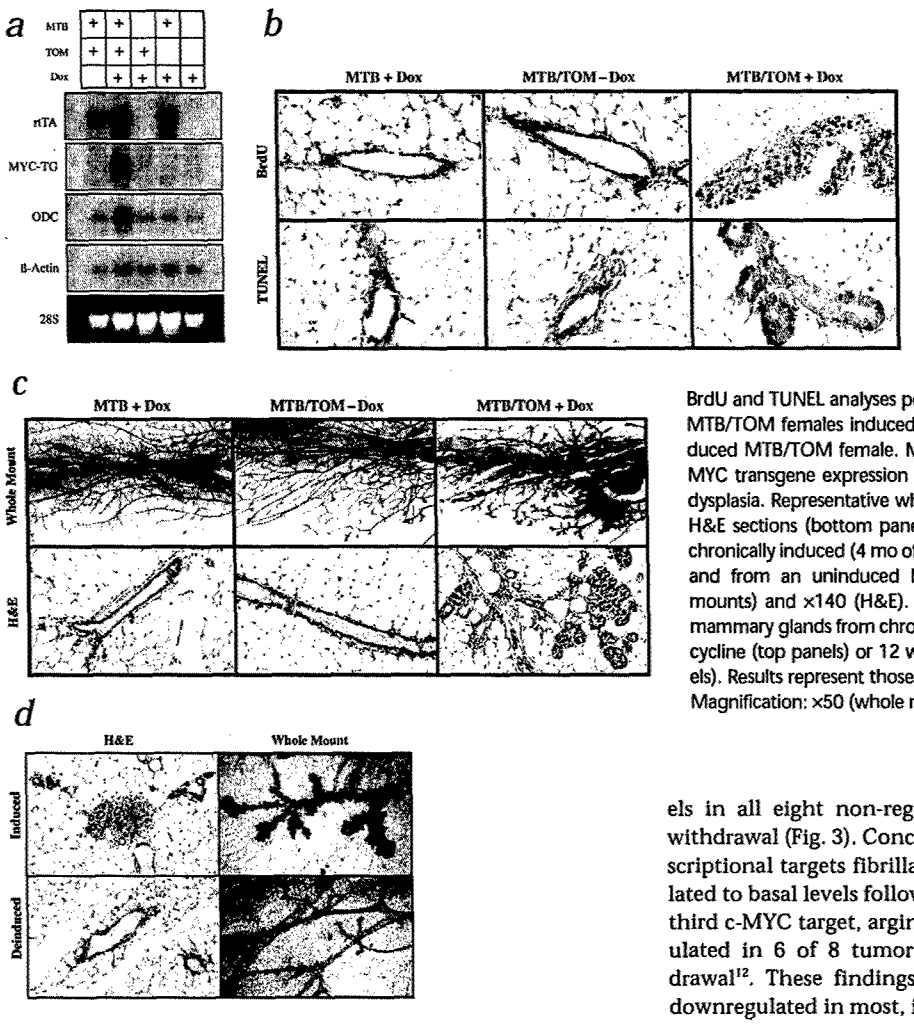

Fig. 1 Chronic induction of c-MYC in the mammary epithelium of MTB/TOM mice results in reversible hyperplastic lesions. ***a***, Inducible transgene expression in MTB/TOM females is tightly regulated and encodes functional c-MYC. Duplicate northern blots containing mammary gland RNA from 8-wk-old female nulliparous FVB wild-type mice or age-matched mice bearing MTB, TOM, or MTB/TOM transgenes. Induced mice (Dox+) were administered doxycycline at 2 mg/ml in their drinking water for 30 d. ***b***, Induced MTB/TOM mice have elevated levels of mammary epithelial proliferation and apoptosis. Representative BrdU and TUNEL analyses performed on mammary sections from MTB and MTB/TOM females induced with doxycycline for 30 d and from an uninduced MTB/TOM female. Magnification: ×300. ***c***, Chronic induction of c-MYC transgene expression results in mammary epithelial hyperplasia and dysplasia. Representative whole mounts (top panels) and hematoxylin and H&E sections (bottom panels) of the #4 mammary gland harvested from chronically induced (4 mo of doxycycline) female MTB and MTB/TOM mice, and from an uninduced MTB/TOM control. Magnification: ×5 (whole mounts) and ×140 (H&E). ***d***, Representative H&Es and whole mounts of mammary glands from chronically induced MTB/TOM mice either on doxycycline (top panels) or 12 wk following doxycycline removal (bottom panels). Results represent those observed in 19 mammary glands from 8 mice. Magnification: ×50 (whole mount) and ×300 (H&E).

mors. Histological analysis of tumors from MTB/TOM females revealed invasive mammary adenocarcinomas similar to those previously described in MMTV-*Myc* transgenic mice[9,10] (Fig. 2*a*).

To determine whether c-MYC overexpression is required for the maintenance of mammary adenocarcinomas, we withdrew doxycycline from chronically induced, tumor-bearing MTB/TOM mice. In a subset of tumors, doxycycline withdrawal resulted in the rapid regression and clinical disappearance of invasive mammary adenocarcinomas (median time to disappearance was 14 d) indicating that maintenance of these tumors is dependent on *MYC* transgene overexpression (Fig. 2*b*). In contrast, other tumors continued to grow following doxycyline withdrawal or decreased in size to a variable extent and then resumed growth.

The fact that a subset of c-MYC-induced tumors grow in the absence of doxycycline indicates that additional genetic alterations have occurred that permit growth in the absence of c-MYC overexpression. Alternatively, the doxycycline-independent growth of such tumors could be due either to doxycycline-independent induction of the *MYC* transgene or to compensatory activation of c-Myc transcriptional targets by endogenous c-Myc. To investigate this, we compared gene expression levels for the *MYC* transgene, endogenous c-Myc and c-MYC transcriptional targets in non-regressing tumors before and after doxycycline withdrawal (Fig. 3). This analysis revealed that *MYC* transgene expression was reduced to undetectable levels in all eight non-regressing tumors following doxycycline withdrawal (Fig. 3). Concordantly, expression of the c-MYC transcriptional targets fibrillarin and SAH-hydrolase was downregulated to basal levels following doxycyline withdrawal[11] (Fig. 3). A third c-MYC target, arginosuccinate synthetase-1, was downregulated in 6 of 8 tumor samples following doxycycline withdrawal[12]. These findings indicate that the c-MYC pathway is downregulated in most, if not all, non-regressing tumors following doxycycline withdrawal. Endogeneous c-MYC expression levels increased following doxycycline withdrawal but were lower than induced levels of c-MYC transgene expression (Fig. 3). As c-MYC represses its own transcription[12], upregulation of endogenous *Myc* in this context is consistent with the overall decreased activation of the c-MYC pathway (Fig. 3). These data indicate that the failure of tumors to regress following doxycycline withdrawal does not result from failure of *MYC* transgene downregulation, or from compensatory upregulation of the endogenous c-MYC pathway.

The long latency period and stochastic formation of tumors in MTB/TOM mice indicated that additional mutations were likely to be required for tumorigenesis. As forced co-overexpression of Hras1 and Myc in the mammary glands of transgenic mice has been shown to accelerate mammary tumorigenesis[13,14], we examined c-MYC-induced mammary tumors for spontaneous *Hras1* mutations. Exons 1 and 2 of *Hras1* were amplified and sequenced from mammary tumors arising in MTB/TOM mice. We detected no mutations in *Hras1* among 47 tumors. Since the *ras* family members, *Kras* and *Nras*, are also mutated in human cancers, we examined these genes for activating point mutations. We found that 49% (23/47) of tumors harbor activating point mutations in either codons 12 or 61 of *Kras2* or *Nras* (*Kras2* versus *Hras1*, $P = 1 \times 10^{-6}$; *Nras* versus *Hras1*, $P = 0.012$). Approximately three quarters of *ras* mutations occurred in *Kras2* (17/23), with the remainder occurring in *Nras* (6/23) (*Kras2* versus *Nras*, $P = 0.008$). We

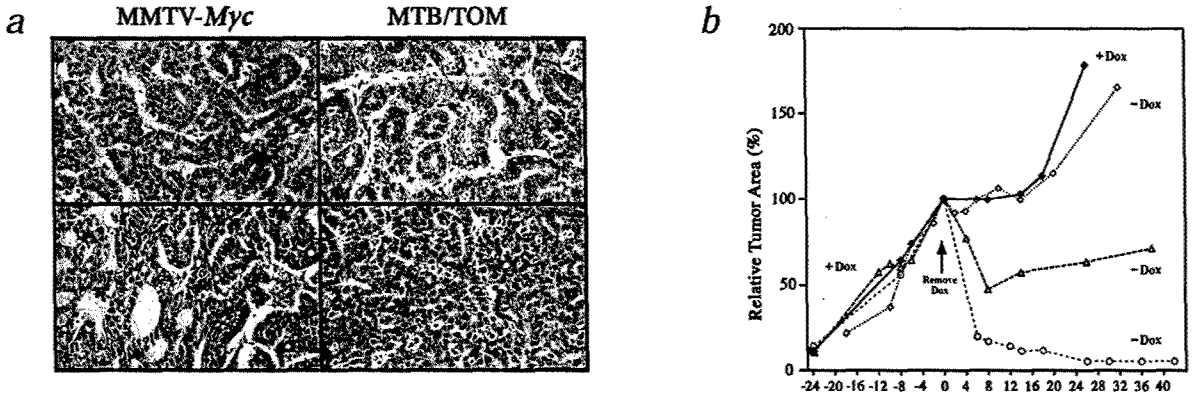

Fig. 2 *MYC* transgene expression is required for maintenance of established mammary tumors. ***a***, Chronic induction of *MYC* transgene expression results in mammary adenocarcinomas. H&E sections of tumors from MMTV-*Myc* and induced MTB/TOM mice. Magnification: ×500. ***b***, Graph showing representative tumor regression patterns following transgene deinduction in three independent chronically induced MTB/TOM females (○,△ and ◇). A control tumor (◆) from a mouse maintained continuously on doxycycline is shown. One tumor (○) underwent full regression to a clinically undetectable state 25 d after doxycycline removal. A second tumor partially regressed during the first 10 d after doxycycline withdrawal, plateaued (△) then resumed growth. Growth of a third tumor (◇) showed essentially no regression.

detected no mutations in *Kras2*, *Hras1* or *Nras* in hyperplastic mammary glands from MTB/TOM mice induced with doxycycline for up to 60 days ($n = 9$) or in mammary glands from wild-type mice ($n = 5$).

To confirm these results, we examined mammary adenocarcinomas arising in MMTV-*Myc* transgenic mice for mutations in *ras* family members. This analysis revealed that 44% (8/18) of Myc-induced tumors harbored detectable *Kras2* mutations in codons 12 or 61, whereas we saw no mutations in *Hras1* or *Nras* (*Kras2* versus *Hras1* and *Kras2* versus *Nras*, $P < 0.001$). Thus, analysis of two independent mouse models for c-MYC–induced carcinogenesis strongly indicates that deregulated expression of c-MYC in the mammary gland selects for spontaneous activation of the *ras* pathway *in vivo*.

To investigate whether tumor regression in this model system is affected by the presence of *ras*-activating mutations, we determined the extent of tumor regression in a panel of 24 primary mammary adenocarcinomas, 15 of which had detectable *ras* mutations and 9 of which did not. We observed complete regression to a clinically undetectable state in 7 of 9 tumors lacking detectable *ras* mutations, but not in any of the 15 tumors bearing *ras* mutations (χ-square, $P = 0.0005$). Three tumors bearing *ras* mutations showed essentially no regression, whereas the remaining twelve tumors bearing *ras* mutations exhibited partial regression, reached a plateau and then resumed growth. Two tumors lacking *ras* mutations did not regress fully. This might be due to failure to detect existing *ras* mutations as a result of heterogeneity within the tumor, to activation of the *ras* pathway by other mechanisms or to alterations in other pathways. As the failure of MYC-induced tumors to regress following doxycycline withdrawal was not due to compensatory activation of c-MYC transcriptional targets by endogenous c-Myc, these data indicate that *ras* activation may abrogate tumor dependence on c-MYC overexpression for growth.

Here we have shown that deregulated expression of c-MYC in the mammary gland strongly selects for spontaneous activation of the *ras* pathway *in vivo* and that these activating mutations accompany the progression of tumors to a state that is no longer dependent on c-MYC for growth. This finding is the first example of spontaneous, recurrent activating mutations in a transgenic model for carcinogenesis. Inducible expression of c-MYC or BCR-ABL in lymphoid tissues and of activated *Hras1* in melanocytes has been shown to result in tumors in transgenic mice, that are capable of regression when transgene expression is abrogated[15–17]. In contrast, reversible effects of oncogenes in epithelial cells *in vivo* have been described only in hyperplasias of the skin and salivary gland[18,19]. Long-term expression of T antigen in the salivary gland eventually results in hyperplasias that are not reversible, although the nature of the genetic changes responsible for this altered behavior is unclear[19]. We now extend these findings by showing that reversal of a single genetic lesion can be sufficient to reverse a common epithelial malignancy in

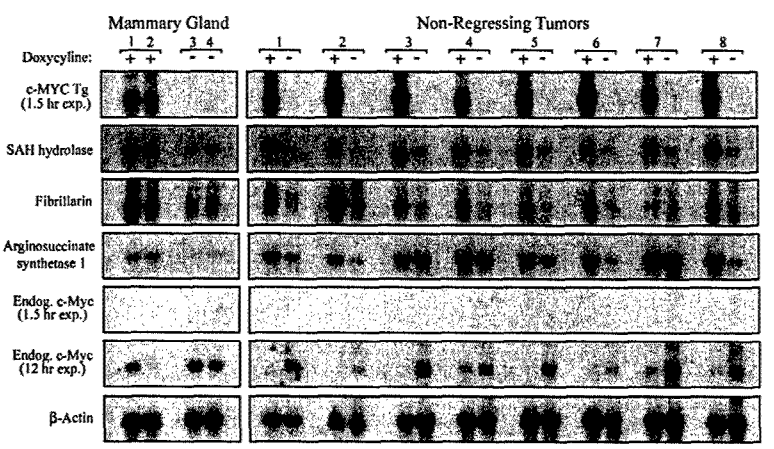

Fig. 3 Non-regressing tumors downregulate c-Myc pathways. Duplicate nothern blots containing either mammary tissue from doxycycline-induced or uninduced MTB/TOM mice or tumor tissue from doxycycline-induced or de-induced mice as indicated. Tissue from eight independent non-regressing tumors was collected before transgene de-induction by biopsy of actively growing tumors in mice on doxycycline (+), or in non-regressing tumors following doxycycline withdrawal (–).

the vast majority of cells, and by identifying a specific secondary mutation that accompanies the progression of tumors to a state independent of the initiating oncogenic stimulus.

The observation that coexpression of c-MYC and *Hras1* can accelerate malignant transformation *in vitro* and *in vivo* constituted an early demonstration of synergy between oncogenic pathways[9,13,20]. However, although these studies proved that the synergistic action of c-MYC and activated *Hras1* is sufficient to accelerate the process of neoplasia, they did not address the question of whether the coexistence of such mutations represents a preferred pathway for tumorigenesis *in vivo*. Our finding that deregulated expression of c-MYC in the mammary gland strongly selects for spontaneous activating mutations in *ras* family members demonstrates that in the setting of a particular primary oncogenic stimulus, mammary tumorigenesis proceeds by the preferential activation of specific secondary oncogenic pathways.

The degree of specificity described here for the occurrence of spontaneous mutations in *Kras2* and *Nras*, as compared with *Hras1*, was unexpected given the previously demonstrated synergy between *Hras1* and c-MYC in mammary tumorigenesis in transgenic mice, and given the high degree of structural and functional homology among the proteins encoded by these genes. Our observations indicate that in the context of c-MYC overexpression in the mammary gland, activating mutations in *Hras1* and *Kras2* might not be functionally equivalent. This conclusion is consistent with the observation that particular *ras* family members are preferentially mutated in different types of human cancer, and by the recent demonstration of differences in the transcriptional targets of these genes[21,22]. Alternately, the specificity observed for Kras2 mutation may reflect higher levels of Kras2 expression in the mammary gland compared to Hras1 and Nras. Notably, activating mutations in *Kras2* have been reported in human primary breast cancers, though infrequently[23]. Potential explanations for the low level of *ras* mutations observed in human breast cancers are that the *ras* pathway is activated by other mechanisms or that such mutations are restricted to a particular subset of tumors. As such, our data indicate that it will be important to determine whether *Kras2* or *Nras* mutations are more common in breast cancers that have amplified the *MYC* locus.

Full regression of mammary adenocarcinomas in MTB/TOM mice strongly correlated with the absence of detectable *ras* mutations. This indicates that therapeutic targeting of c-MYC may be an effective treatment approach only for c-MYC-induced breast cancers in which the *ras* pathway has not been activated. However, as in other inducible transgenic model systems, a fraction of the c-MYC-induced tumors in our model system that had fully regressed following transgene de-induction recurred after several weeks or months. This indicates that tumor heterogeneity might permit the emergence of neoplastic cells that are no longer dependent upon the c-MYC transgene for growth. Our observations that c-MYC overexpression selects for spontaneous mutations in *ras* family members *in vivo*, and that *ras* mutations accompany tumor progression to a transgene-independent state, suggest that identifying specific sets of preferentially associated oncogenic mutations will facilitate targeting of the multiple synergistic pathways that contribute to neoplastic growth.

Methods

Transgenic mice. We generated the pMMTV-rtTA expression vector by cloning the 1.1 kb *Eco*RI/*Bam*HI fragment encoding rtTA from pUHD172-1neo (ref. 3) into pBS-MMTV-pA, which consists of a plasmid BlueScript backbone (Stratagene, La Jolla, California), the MMTV-LTR upstream of the *Hras1* leader sequence and a multiple cloning site directly upstream of the SV40 splice site and polyadenylation signal (E.J. Gunther, submitted). The pTetO-MYC expression vector was generated by cloning exons 2 and 3 of human *MYC* from pSV7Humyc (ref. 4) into pTet-Splice (Gibco BRL, Life Technologies, Rockville, Maryland). For each construct, founder lines were generated by injecting linearized plasmid DNA into fertilized oocytes collected from superovulated FVB mice. Transgenic mice and littermate controls were administered doxycyline (0.5–2.0 mg/ml) in their drinking water. Mice were monitored twice per week for tumor formation. Calipers were used to measure tumor area in two dimensions. Mice bearing tumors were biopsied to obtain tumor tissue before doxycycline withdrawal. After approximately 1 wk, doxycycline was withdrawn from the drinking water of biopsied mice and tumors were monitored for regression behavior. First-strand cDNA was prepared from tumor material collected at biopsy and exons 1 and 2 of *Hras1*, *Kras2* and *Nras* were amplified by PCR and sequenced to detect point mutations.

Northern-blot hybridization. We performed total RNA isolation and northern hybridization as described[24] using 3 µg of total RNA from snap-frozen tumors or mammary tissue from which the lymph node in the number four mammary gland had been removed. Blots were hybridized with cDNA probes for a 360 bp fragment of rtTA (nt 1441–1800), exons 2 and 3 of human *MYC*, *ODC* (nt 725–1204), *FBL* (nt 39–540; encoding fibrillarin), *SAHH* (nt 249–801), *ASS* (nt 45–918), exon 1 of mouse *Myc*, or *Actb*. c-MYC transgene and endogenous *MYC* were detected using probes of approximately equal specific activity to facilitate comparison of expression levels.

Immunohistochemistry. We injected mice with 1 mg BrdU per 20 g body weight 2 h before killing. Mammary gland #4 was removed and fixed overnight in neutral buffered formalin, transferred to 70% ethanol and embedded in paraffin. 5 µm sections on ProbeOn Plus (Fisher Pittsburgh, Pennsylvania) slides were dewaxed in Xylene, then sequentially rehydrated in 100%, 95%, and 70% ethanol, followed by PBS. Sections were pretreated in 2 M HCl for 20 min at room temperature, washed in 0.1 M Borate buffer pH 8.5 x 2, and rinsed in PBS. BrdU immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector, Burlingame, California), rat IgG antibody against BrdU (Accurate Lab, Westbury, New York) and a secondary biotinylated rabbit antibody against rat IgG according to manufacturer's instructions. Sections were counterstained for 10 min in 0.5% (w/v) methyl green in 1.0 M NaOAc, pH 4.0.

TUNEL Analysis. We performed TUNEL analysis using the Apoptag Peroxidase Kit (Intergen, Purchase, New York) according to the manufacturer's instructions. Sections were pretreated in Proteinase K (20 µg/ml) for 15 min at room temperature, washed in de-ionized water twice for 2 min each, incubated in equilibration buffer, then incubated at 37 °C for 1 h with a 1:10 dilution of TdT enzyme in 1x reaction buffer. Reactions were terminated, developed using anti-digoxigenin-alkaline phosphatase Fab fragments (Roche) and nitroblue tetrazolium chloride per manufacturer's instructions, and counterstained in methyl green. Images from BrdU and TUNEL sections were captured digitally and areas of positively stained and unstained nuclei were quantitated by color segmentation analysis using Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland). Quantitative analysis was performed on 4–8 fields per section consisting of approximately 2,500–10,000 cells.

Whole mounts. We spread #4 mammary glands on glass slides and fixed for 24 h in 10% neutral buffered formalin. Glands were placed in 70% ethanol for 15 min followed by 15 min in deionized water before staining in 0.05% carmine/0.12% aluminum potassium sulfate for 24–48 h. Glands were dehydrated sequentially in 70%, 90% and 100% ethanol for 10 min each and then cleared in toluene or methyl salicylate overnight. For histological analysis, mammary glands were fixed as above and transferred to 70% ethanol prior to paraffin embedding. 5 µm sections were cut and stained with hematoxylin and eosin.

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